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NOTES ON THE DETERMINATION OF *B. COLI* IN WATER.*

FRANCIS F. LONGLEY AND WARREN U. C. BATON.

(From the Laboratories of the Filtration Plant, Washington, D. C.)

THE best laboratory evidence of the sanitary quality of a water supply is to be obtained from a study of the bacterial flora of the water, with special reference to organisms which may have had their origin in sewage.

The most typical of these sewage organisms, and the one most frequently looked for as an index of sewage pollution, is the colon bacillus.

The number of these organisms in a water supply has a considerable range, from a few hundred per c.c. in a grossly polluted water, down to practically none in some springs and deep wells, surface water under occasional conditions, and the effluent of a filter of high efficiency. When they are frequently present, the determination of the number of acid-forming colonies developing upon the litmus lactose agar plate of Wurtz gives an indication sufficient for all practical purposes of the polluted condition of the water. When they are quite absent from a water it is a simple matter to demonstrate the fact with reasonable certainty. The complications and the refinements in the determination of the presence of this organism are brought out in a long-continued routine examination of waters which lie between the two limiting classes, that is, in which the most delicate tests show *B. coli* to be sometimes absent and sometimes present, but in numbers too small for direct determination. Especially is refinement in this work necessary in determining the sanitary efficiency of a filter, under conditions which may bring forth critical inspection of the results obtained.

In the work of this laboratory we have accumulated a mass of data obtained in carefully conducted coli work, and we present it here in statistical form, with certain observations upon the various factors of the work, for the benefit of others engaged in similar studies.

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* Received for publication March 25, 1907.

The work has been done entirely along the lines laid down in the *Report of the Standard Committee on Water Analysis of the American Public Health Association*, supplemented by various other means for gaining as broad a knowledge as possible of the work in hand.

In order to permit a more general interpretation of certain parts of the work presented herein, we have obtained data on gas formation on several different classes of waters, including surface waters in other localities and under other conditions of pollution, lake waters, and well waters. These tend, without exception, to substantiate our conclusions regarding the points in question.

For certain of these data, and for suggestions and criticisms in the preparation of this paper, we take this occasion to express our appreciation and thanks to Mr. Geo. C. Whipple, New York; and Mr. Karl F. Kellerman, of the Bureau of Plant Industry, Department of Agriculture, Washington, D. C.

OUTLINE OF WORK.

The discussion of the several parts of this work is taken up according to the following outline—

(1) GAS FORMATION.
  (*a*) Quantity of gas produced by *B. coli.*
  (*b*) Quantity of CO_2 produced by *B. coli.*
  (*c*) Diagnostic value of the fermentation test.
(2) LENGTH OF PERIOD FOR PRELIMINARY FERMENTATION.
(3) VALUATION OF SUBCULTURE TESTS.
(4) THE PRESUMPTIVE GAS TEST, AND ITS APPLICATION TO THE ROUTINE EXAMINATION OF POTOMAC WATER, IN COMPARISON WITH A COMPLETE CONFIRMATORY TEST.
(5) OTHER PRESUMPTIVE TESTS.
  (*a*) Wurtz litmus lactose agar plate.
  (*b*) Neutral red reaction.
  (*c*) Use of the bile salts.

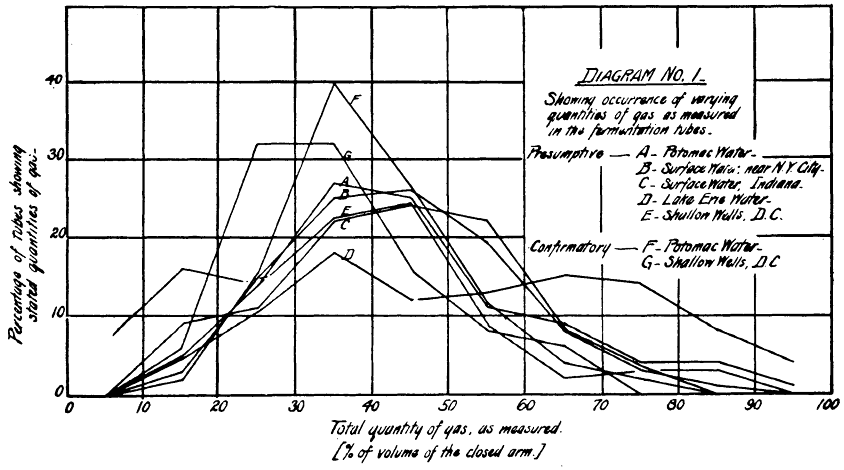
(1) GAS FORMATION.

Of all the cultural reactions for the identification of *B. coli*, one of the most important is the formation of gas in dextrose broth. It has been generally taught that if the total amount of gas measured in the fermentation tube is from 25 to 70 per cent of the capacity of the closed arm; and of this gas, approximately two-thirds is hydrogen and one-third carbon dioxide, the test is considered typical. If the amount of gas is greater than 70 per cent or less than 25 per cent, but not less than 10 per cent, or if there is an amount of CO_2 greater than 40 per cent of the total gas, the test is considered atypical. If the quantity of gas is less than 10 per cent the test is negative.

This statement of quantities of gas and CO_2 is applied most specifically to the presumptive gas test for *B. coli.*

Authorities have apparently avoided stating any definite limits for gas production and CO_2 in the subculture fermentation tube which forms a part of the confirmatory test, dismissing the subject with the very general statement that *B. coli* ferments certain carbohydrate broths with the formation of about 50 per cent of gas, of which about one-third is CO_2. There is good reason for the omission of a definite statement on this point.

The quantities of gas and CO_2 are often very erratic, even in pure culture, though a process of rejuvenation will often restore the typical gas formation. The fermentation of sugar broths is the only one of the subculture tests which is of a quantitative nature, and it is unfortunate that more significance can not be attached to this. Our experience, however, does not bring us any nearer to this end.



Consideration of this point will be taken up under two heads, namely: (*a*) Quantity of gas; (*b*) Quantity of CO_2.

(*a*) *Quantity of gas.*—We have tabulated the gas formation, classified according to the percentage of total gas measured, in routine coli work on the Potomac water, and also on other classes of water. The gas formation in the preliminary or presumptive tubes, and in the subculture or confirmatory tubes, are tabulated separately. Diagram 1 shows this distribution more graphically. These tables and curves are quite typical and need no comment, except to point out the greater maximum and the narrower limits in the case of the confirmatory gas formation.

TABLE 1.

(*a*) PERCENTAGE OF PRESUMPTIVE FERMENTATION TUBES SHOWING THE STATED QUANTITIES OF GAS.

Class of water	No. of Observations	Total Quantity of Gas Between									
		0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100
Potomac water	452	0.2	5	14	27	25	11	9	4	4	1
Surface water near New York City	552	0	3	15	25	26	19	8	3	1	0
Surface water—Indiana	96	0	9	11	22	24	22	8	4	0	0
Lake Erie water	175	0	5	11	18	12	13	15	14	8	4
Shallow wells, Washington, D. C.	98	7	16	14	22	24	9	2	3	3	0

(*b*) PERCENTAGE OF CONFIRMATORY FERMENTATION TUBES SHOWING THE STATED QUANTITIES OF GAS.

Class of water	No. of Observations	0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100
Potomac water	528	0	2	15	40	26	11	4	2	0	0
Shallow wells, Washington, D. C.	50	0	6	32	32	16	8	6	0	0	0

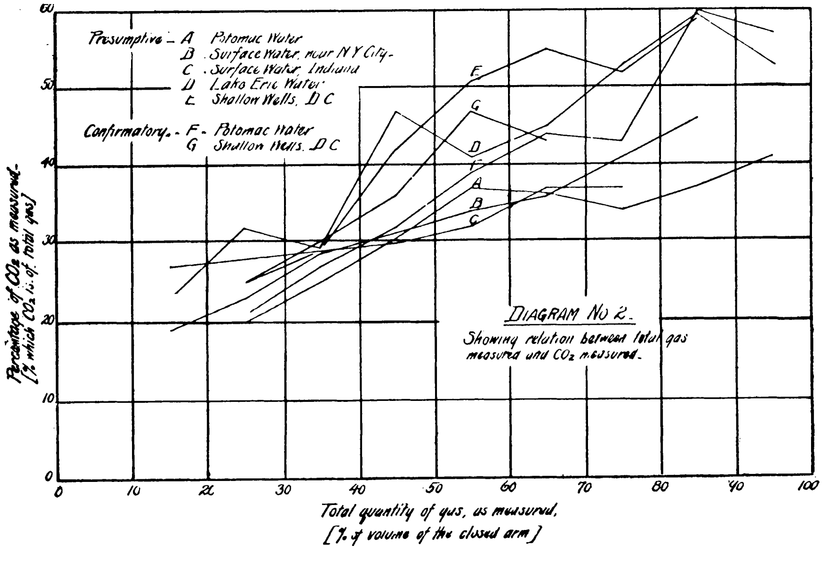


TABLE 2.

(*a*) AVERAGE PERCENTAGE OF CO_2 PRESENT IN PRESUMPTIVE FERMENTATION TUBES SHOWING STATED QUANTITIES OF GAS.

Class of Water	No. of Observations	Total Quantity of Gas Between									
		0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100
Potomac water	452	..	..	20	25	31	37	36	34	37	41
Surface water, near New York City	552	..	19	23	29	31	34	36	41	46	..
Surface water, Indiana	96	..	27	28	29	30	32	37	37	..	..
Lake Erie water	175	..	23	32	29	47	41	45	53	60	53
Shallow wells, Washington, D. C.	98	..	..	25	29	42	51	55	52	59	..

(*b*) AVERAGE PERCENTAGE OF CO_2 PRESENT IN CONFIRMATORY FERMENTATION TUBES SHOWING STATED QUANTITIES OF GAS.

Potomac water	528	..	..	21	27	32	39	44	43	60	57
Shallow wells, Washington, D. C.	50	..	..	25	30	36	47	43	..	..	..

TABLE 3.

(*a*) AVERAGE PERCENTAGE OF CO_2 PRESENT IN PRESUMPTIVE FERMENTATION TUBES SHOWING STATED QUANTITIES OF GAS

Cultures from Potomac Water	No. of Observations	Total Quantity of Gas Between									
		0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100
Not producing nitrites	39	..	..	25	30	34	31	36	..	32	..
Not producing indol	97	..	..	15	29	34	37	37	34	38	..
Miscellaneous gas formers	69	..	..	24	27	33	35	44	45	46	39

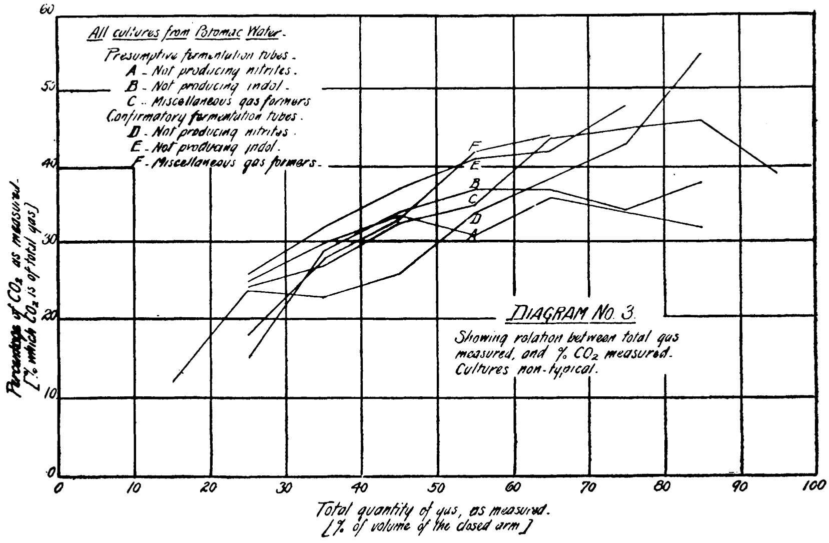


DIAGRAM No. 3.
Showing relation between total gas measured, and % CO_2 measured. Cultures non-typical.

(*b*) AVERAGE PERCENTAGE OF CO_2 PRESENT IN CONFIRMATORY FERMENTATION TUBES SHOWING STATED QUANTITIES OF GAS.

Not producing nitrites	40	..	12	24	23	26	34	..	43	55	..
Not producing indol	106	..	..	26	32	37	41	42	48	..	..
Miscellaneous gas formers	76	..	..	18	28	33	42	44	..	..	..

(*b*) *Quantity of* CO_2.—It has been generally stated that the quantity of CO_2 from a typical culture of coli, as determined by absorption by sodium hydrate, should be about one-third of the total volume of gas produced. This of course has been looked upon as only an approximate relation, and some latitude has been permitted in its application. We have long noticed, however, that this relation did not hold, but that there was instead a more or less regular variation in percentage of CO_2 depending on the total quantity of gas present.

We have therefore made a statistical analysis of our accumulated data with special reference to this relation between gas and CO_2, and in the same tables we give similar figures for the other classes of waters.

In Diagram 2 these relations are shown graphically to better advantage. It will be seen that there is an unmistakable increase in the percentage of CO_2 measured, with an increase in the total quantity of gas. Some of the curves are quite irregular, and there seems to be a tendency for the curve to fall off as the total quantity of gas approaches 100 per cent. This does not interfere with the general conclusion, however.

The relation we have pointed out does not seem to have any diagnostic value. As will be explained later we think it is a purely physical phenomenon, which perhaps obscures, rather than renders plainer, the significance of a quantitative gas test. The increase of CO_2 with increased total gas is not peculiar to *B. coli*, but seems to be true generally of gas-forming organisms. This is evident from the figures given in Table 3, and from Diagram 3, which is merely an extension of the analysis of our data to include the atypical forms of *B. coli* that fail to produce nitrites and indol, and a miscellaneous lot of gas-producing cultures that are entirely without the coli classification.

No theory has ever come to our attention to explain the variable relations between gas and CO_2; nor have we been able to suggest any theory that would account for the *production* of varying proportions of the constitutent gases, by organisms of one kind, under quite constant conditions. It has been suggested as a much more probable theory that the gas as *produced* might have a more or less constant composition; and that the explanation for the variations we have observed might be found in some physical phenomena connected with the incubation of the cultures.

The gas produced in the fermentation tube by *B. coli* and other organisms of that general type probably consists of CO_2, hydrogen, nitrogen, methane, and perhaps other light hydro-carbons. CO_2 is readily absorbed in water, while hydrogen and other light gases are scarcely absorbed at all, the quantity being less than 5 per cent of the quantity of CO_2 that could be absorbed at the incubator temperature of 40° C., other conditions being equal. Therefore with the small quantities of gas to be measured, and the rather crude methods of measurement in routine work, the absorption of gases other than CO_2 may be neglected.

Under the conditions of incubation, the temperature and pressure of the gases in the closed arm of the fermentation tube and also the area of liquid exposed to absorption are substantially constant. Then leaving out of consideration other refinements of this problem, the actual quantities of CO_2 absorbed in different tubes will probably be about equal. The length of time the gases are exposed for absorption, from the time they begin to form until they are measured, may have an influence upon the quantity absorbed, and may account for some of the observed irregularities; but in general, it is probable that about equal quantities of CO_2 will be retained in solution in different fermentation tubes.

If this be so we will have the following conditions:

(*a*) A small quantity of total gas of approximately constant composition as produced; an approximately constant quantity of CO_2 absorbed, or held in solution as fast as formed depending upon temperature, pressure, etc.; and consequently a relatively small quantity or percentage of CO_2 remaining to be measured by absorption with sodium hydrate.

(*b*) A large quantity of total gas of same composition as above;

same constant quantity of CO_2 absorbed or held in solution; and consequently a relatively large quantity or percentage of CO_2 remaining to be measured by absorption with sodium hydrate.

And similarly for other quantities of total gas within or beyond the range of the cases here given; so that in accordance with this theory we should expect to find CO_2 present above the liquid in the fermentation tubes in percentage quantities varying with the total quantities of gas present.

This can be put in mathematical form. Let "*a*" be the approximately constant quantity of CO_2 absorbed under the given conditions of temperature and pressure expressed as the percentage of the total volume of the closed arm.

Assume that the percentage of CO_2 actually produced is 75 per cent, which is approximately the figure we have found under the most favorable conditions, as will be shown further on.

Then we may make a table as follows:

TABLE 4.

Total Quantity of Gas		Residual Gases =25 Per Cent	Percentage of CO_2 as Measured	When $a=$		
Measured	Produced			40%	50%	60%
10	$10+a$	$0.25\,(10+a)$	$\frac{10-0.25\,(10+a)}{10}$	..	..	..
20	$20+a$	$0.25\,(20+a)$	$\frac{20-0.25\,(20+a)}{20}$	25	12½	..
30	$30+a$	$0.25\,(30+a)$	$\frac{30-0.25\,(30+a)}{30}$	42	33	25
40	$40+a$	$0.25\,(40+a)$	$\frac{40-0.25\,(40+a)}{40}$	50	44	37
50	$50+a$	$0.25.(50+a)$	$\frac{50-0.25\,(50+a)}{50}$	55	50	45
60	$60+a$	$0.25\,(60+a)$	$\frac{60-0.25\,(60+a)}{60}$	58	54	50
70	$70+a$	$0.25\,(70+a)$	$\frac{70-0.25\,(70+a)}{70}$	61	57	53
80	$80+a$	$0.25\,(80+a)$	$\frac{80-0.25\,(80+a)}{80}$	63	59	56
90	$90+a$	$0.25\,(90+a)$	$\frac{90-0.25\,(90+a)}{90}$	64	61	58
100	$100+a$	$0.25\,(100+a)$	$\frac{100-0.25\,(100+a)}{100}$	65	63	60

This table is shown diagramatically, and the relations between total quantity of gas measured, and total percentage of CO_2 measured are entirely in accordance with the facts, if the above assumptions

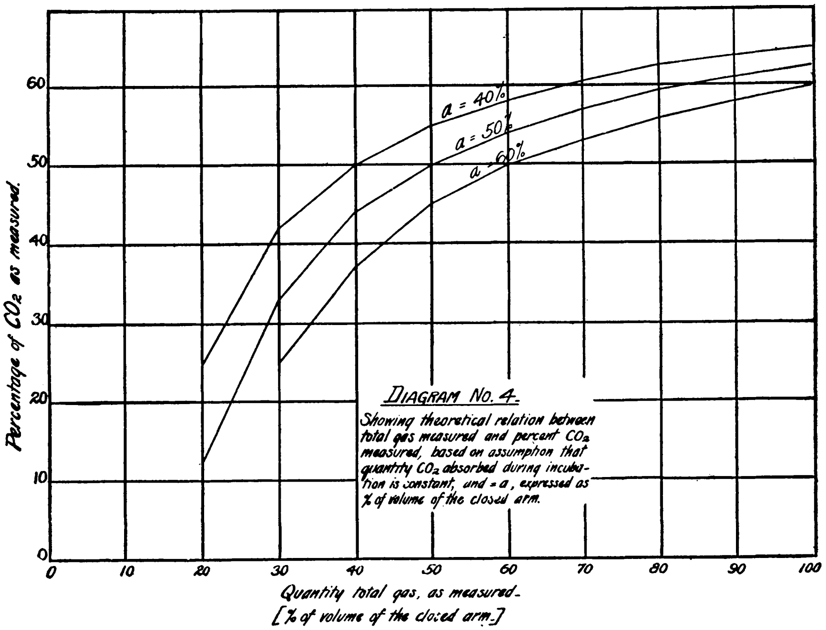


DIAGRAM NO. 4.
Showing theoretical relation between total gas measured and percent CO_2 measured, based on assumption that quantity CO_2 absorbed during incubation is constant, and = a, expressed as % of volume of the closed arm.

are not unreasonable. This is true, not only for the cultures of typical *B. coli*, as determined by complete confirmatory tests, but also, with rather more irregularities, for the atypical or paracolon forms, for the miscellaneous gas formers, and for the presumptive tests on the other classes of water. This would seem to indicate that for a great many of the gas-forming organisms ordinarily found in water supplies, the composition of the gas so formed was more or less constant.

In accordance with this theory we should expect to find, too, some constant relation between total gas and CO_2 if the tubes were incubated under conditions that would prevent the absorption by the media of any of the CO_2 as formed. Efforts were made to produce these conditions.

The closed arms of the fermentation tubes were filled with CO_2 in such a manner as to preserve their sterile condition, after which the tubes were placed under pressure in an atmosphere of CO_2 for a time. The CO_2 remaining unabsorbed in the tubes was then removed, and they were then inoculated with pure cultures of *B. coli* and incubated for 48 hours at 40° C. in an atmosphere of CO_2 at atmospheric pressure. Control tubes were inoculated and incubated each time under ordinary atmospheric conditions. The results of these experiments are given below. Two classifications are given for the tubes charged with CO_2; "A" being the last we have carried through, charged under somewhat more perfect conditions than "B," which represent the earlier work. "C" represents the control tubes, corresponding to the ordinary conditions under which fermentation tubes are incubated.

	A	B	C
Maximum percentage CO_2.....	78	63	40
Minimum " "	70	40	12
Average " "	75	49	30

It was proven by careful experiments that the high percentage of CO_2 obtained under these conditions was not due to the liberation of the CO_2 used in charging the tubes under the physical conditions of incubation.

We do not feel satisfied that we have produced conditions perfect enough to give us the exact CO_2 content; but the evidence seems to indicate clearly that for the class of cultures we have made use of in these experiments the percentage of CO_2 actually produced is approximately constant, and is probably at least 75 per cent of the total quantity of gas produced. And from the marked similarity of the curves showing the relations between gas and CO_2, for this class of cultures and for all the other classes given, it may reasonably be inferred that the same statement is of more or less general application.

(*c*) *Diagnostic value of the fermentation test.*—In a careful consideration of the diagnostic value of the fermentation test, attention must be given separately to its application to the presumptive and to the confirmatory tests, and also to its relative values when used qualitatively or quantitatively.

The presumptive gas test depends for its value entirely upon the principle that a sample of water properly inoculated into fermentation tubes, giving quantities of gas and CO_2 lying within certain limits, prescribed as the result of much careful experience, *most probably* contains *B. coli.* The fermentation test, in its application to the "presumptive test" is of an unquestioned value, and an essential feature of this is that it shall be quantitative.

It is common experience and is borne out by our own data, that the quantity of gas formed in confirmatory fermentation tubes varies within wide limits. We have shown by the foregoing data that the percentage of CO_2 actually produced by *B. coli* and also by other gas-forming organisms, is approximately constant, and probably 75 per cent or more of the total gas; and that the variations from that quantity can in general be explained by the physical phenomenon of absorption of the CO_2 during incubation. We know further that the majority of cultures that show what may be termed "abnormal" quantities of gas and CO_2 in the subculture fermentation tube may be brought to such a condition, by proper methods of rejuvenation, that they will produce gas and CO_2 well within the "normal" limits—that is, approaching more or less closely to "about 50 per cent total gas, of which about one-third is CO_2" as measured by the ordinary methods.

If, then, the quantity of gas produced by a miscellaneous lot of organisms can be controlled, and the percentage of CO_2 is approximately constant, the diagnostic value of a *quantitative* gas test in the determination of the specific organism, *B. coli*, can be but small. As a *qualitative* test in this connection, it is of course of the greatest value.

Generally speaking, however, all the evidence we have seems to indicate that there is no determinative significance attached to the quantities of gas and CO_2 in the confirmatory or subculture fermentation tube.

As we have indicated below, our conclusions on this point have led us to eliminate all observations on gas formation except the simple presence or absence of gas. This permits the elimination also, of the old, cumbersome, expensive, difficultly cleaned fermentation tube, and the use of a gas tube improvised by inverting a small test-tube inside a larger one. We have made successful use of tubes of the following sizes:

Outside tube, 6″ × 1″
Inside tube, 2-3/4″ × 1/2″

The inside tube, which is simply placed in an inverted position in the larger tube, with the latter filled with the necessary quantity of broth, will fill on being taken out of the sterilizer. High steam pressure is not necessary. The tubes will fill equally well in the Arnold and in the autoclave.

This makes the examination of large quantities of water for gas formers a simple operation, too. It is only necessary to invert a small test-tube inside an ehrlenmeyer flask holding sufficient broth to fill the inverted tube on sterilization, and of the proper strength. The sample of water is then added, and incubation gives visibly and unmistakably the presence or absence of gas.

(2) LENGTH OF PERIOD OF PRELIMINARY FERMENTATION.

The exigencies of a laboratory in which a large volume of work is carried on necessitate not only the elimination of unnecessary work, but also economy in such factors as time of incubation of cultures, quantity of glassware in use at one time, incubator space, etc. Some studies have therefore been made to show the value of a 24-hour period as compared with a 48-hour period of incubation of preliminary fermentation tubes.

Out of 1091 preliminary fermentation tubes showing approximately typical gas and CO_2 at the end of 48 hours only 519 tubes, or less than 50 per cent of them, showed quantities of gas falling within the proper limits for positive presumptive test at the end of 24 hours. It is plainly evident from this that a 24-hour period of incubation for the fermentation tube in the presumptive test is not sufficient.

A 24-hour period is sufficient, however, to indicate a negative result in case there is no gas formed at the end of that period, as the following figures will show:

Number of samples showing no gas in 24 hours, but some gas in 48 hours = 173.

Of these proven to contain coli = 2.

If all these 173 samples had been recorded as negative at the end of 24 hours when they did not show any gas, the error would have been only 1 per cent.

We may also conclude from these results, that when all suspected cultures are regularly carried through the complete test for coli, we may after plating from the preliminary fermentation tube to the litmus lactose agar plate at or before 24 hours, discard all the preliminary fermentation tubes, recording as negative all those which contain no gas at 24 hours. By so doing we lose the additional diagnostic value of the readings on gas and CO_2 in preliminary tubes, but these at best are of less value than the results obtained in subculture dextrose broth; and even these latter we have shown to be of but small value.

(3) VALUATION OF SUBCULTURE TESTS.

From an analysis of accumulated data such as we have here, the relative value of the various subculture tests may be determined.

These are values which of course will vary with a great many conditions, as will be seen from the tabulation given below. But the variation is over a comparatively limited range, especially in the case of those subcultural tests subsequent to the litmus lactose agar plate, and therefore the figures given are believed to have a more or less general value.

The test for *B. coli* is throughout a process of elimination. Were we carrying on our routine work entirely by the presumptive tests then we would eliminate by the first gas test all those cultures which, by the law of probabilities, were not positive. In applying the complete test for *B. coli*, however, we may avoid some of the uncertain factors of this application. We eliminate only those cultures which we have the best reasons to believe will be negative and proceed to confirm either positive or negative, by further work, all cultures which up to this point may be looked upon as suspicious.

This valuation of the subculture work is therefore based on a total number of cultures somewhat greater than would be considered probably positive on the strength of the presumptive gas test alone. From the tables it is evident that the greater part of this excess of doubtful cultures is eliminated by the very next test in order, the litmus lactose agar plate.

In these tables we have considered 100 per cent to represent the total number of preliminary fermentation tubes in which there was any gas formation in 24 hours, or if none in 24 hours, then an appreciable amount in 48 hours. The same matter is given in two forms; first, showing separately the values of the various subculture tests on the basis defined above; and second, showing on the same basis the percentage of cultures remaining after elimination by each of the successive subculture tests.

One complication must be noted. A good many cultures are eliminated through the failure of more than one of the tests, and these have been included under one head, as miscellaneous negatives.

TABLE 5.

MONTH	TOTAL NUMBER OF SAMPLES	PERCENTAGE OF SAMPLES GIVING PRELIMINARY FERMENTATION	NUMBER OF SAMPLES GIVING PRELMINARY FERMENTATION	PERCENTAGE OF CULTURES SHOWING PRELIMINARY FERMENTATION, ELIMINATED BY FOLLOWING TESTS:							
				Litmus Lactose Agar Plate	Agar Slant and Microscopical Examination	Miscellaneous Subcult.	Nitrites	Indol	Milk	Gel. Stab	Confirmed. Positive
March........	411	39.4	162	53.1	9.3	7.4	2.5	3.1	2.5	0.6	21.5
April.........	350	58.0	203	41.4	3.5	7.4	5.4	1.0	3.0	0.5	37.8
May..........	432	47.9	207	82.6	1.4	2.9	4.3	1.4	1.4	0.0	6.0
June..........	424	70.5	299	54.2	3.3	10.4	1.7	6.0	3.0	0.0	21.4
July..........	285	79.6	227	47.1	6.2	11.5	4.8	6.2	3.1	2.6	18.5
August........	487	85.6	417	33.3	7.2	4.1	2.2	9.4	1.4	1.2	41.2
September....	278	67.3	187	48.1	14.4	8.0	1.1	8.6	4.8	0.5	14.5
October.......	330	61.8	204	48.5	4.4	7.8	1.5	2.5	10.3	1.5	23.5
November....	275	45.1	124	46.8	9.7	4.8	4.8	16.1	0.0	0.8	17.0
December.....	281	50.2	141	29.1	14.9	10.6	11.3	6.4	0.7	0.7	26.3
Totals....	3553		2171								
Mean values		61.1		47.7	6.8	7.3	3.5	6.0	3.0	0.9	24.8

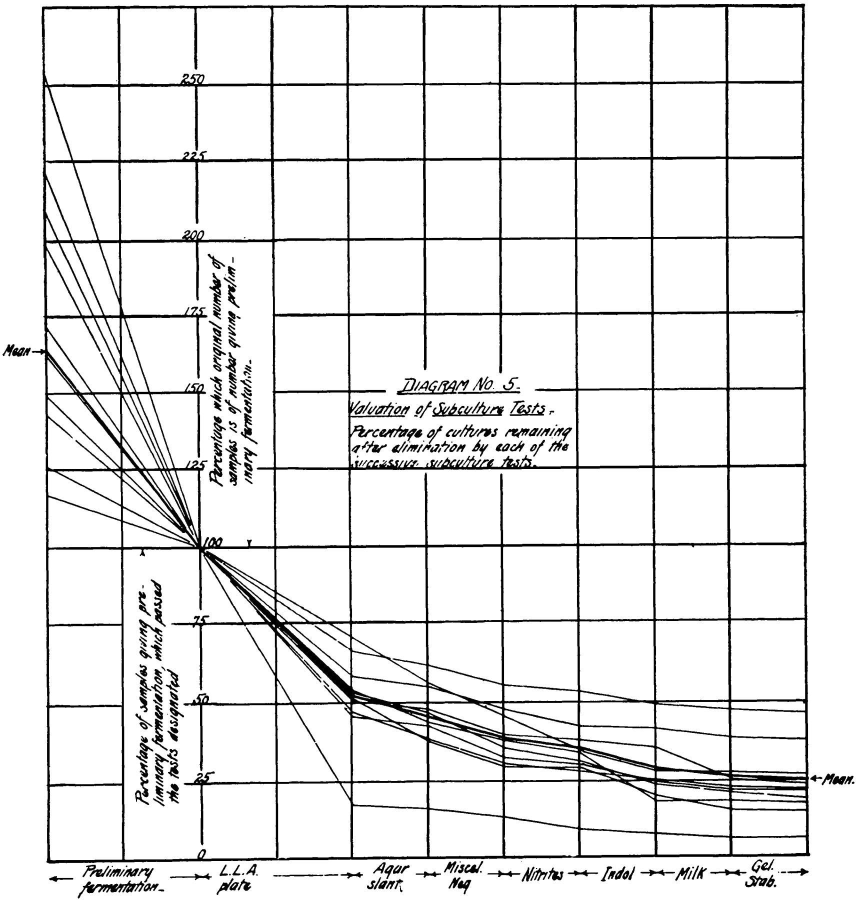


DIAGRAM NO. 5.

Valuation of Subculture Tests.

Percentage of cultures remaining after elimination by each of the successive subculture tests.

TABLE 6.

Month	Samples Giving Preliminary Fermentation	Percentage of Samples Giving Preliminary Fermentation, Passing All Tests to and Including						
		L. L. A. Plate	Agar Slant and Microscop. Examination	Miscellaneous Sub-cult.	Nitrites	Indol	Milk	Gel. Stab
March	100	46.9	37.6	30.2	27.7	24.6	22.1	21.5
April	100	58.6	55.1	47.7	42.3	41.3	38.3	37.8
May	100	17.4	16.0	13.1	8.8	7.4	6.0	6.0
June	100	45.8	42.5	32.1	30.4	24.4	21.4	21.4
July	100	52.9	46.7	35.2	30.4	24.2	21.1	18.5
August	100	66.7	59.5	55.4	53.2	43.8	42.4	41.2
September	100	51.9	37.5	29.5	28.4	19.8	15.0	14.5
October	100	51.5	47.1	39.3	37.8	35.3	25.0	23.5
November	100	53.2	43.5	38.7	33.9	17.8	17.8	17.0
December	100	70.9	56.0	45.4	34.1	27.7	27.0	26.3
Mean values	100	52.3	45.5	38.2	34.7	28.7	25.7	24.8

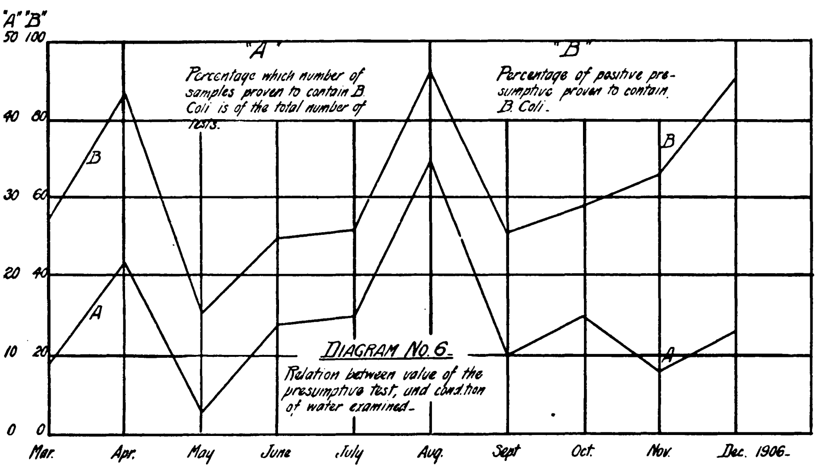


DIAGRAM No. 6.

Relation between value of the presumptive test, and condition of water examined.

These tables and diagrams show that by far the greater part of the elimination of cultures is effected in the preliminary fermentation tube and the litmus lactose agar plate, subsequent eliminations being comparatively small and constant. Therefore the best presumptive test we could use for the conditions in this laboratory would be the preliminary fermentation tube followed by the litmus lactose agar plate.

(4) THE PRESUMPTIVE GAS TEST AND ITS APPLICATION TO THE ROUTINE EXAMINATION OF POTOMAC WATER, IN COMPARISON WITH A COMPLETE CONFIRMATORY TEST.

While the regular procedure of this laboratory in the determination of the presence of *B. coli* is to carry each culture through the full confirmatory tests, it has been recognized as desirable to determine the value of the so-called "presumptive tests" on this water.

There is sometimes a misconception of the proper meaning of the term "presumptive test." A positive test of this kind is often construed to mean that *B. coli* is presumably present. There is not accuracy enough in this presumption. If would be better not to confuse the careful determination of the specific organism *B. coli* with the time and labor-saving determination, by means of this presumptive gas test, of the general character of a water as regards pollution. That the use of this test gives a reasonably accurate idea as to whether or not pollution exists, and in what quantity, has been conclusively proven, and is generally accepted. But the relation between the percentage occurrence of *B. coli*, and the percentage of samples giving presumptive evidence of pollution is very variable, and the one should not be taken for the other.

We have noted each culture during the preliminary cultivation with reference to the fulfillment of the following conditions:

(1) Total gas in 48 hours, between 25 and 70 per cent of the capacity of the closed arm of the fermentation tube.

(2) CO_2 between 20 and 40 per cent of the total gas.

Cultures having the above quantities of gas and CO_2 were considered typical positive presumptive.

Comparison of these figures with the total number of samples determined positive by the complete test is given below:

TABLE 7.

COMPARISON OF PRESUMPTIVE AND CONFIRMATORY TESTS ON POTOMAC WATER

1906	Total Separate Presumptive Tests	Total Positive Presumptive	Total Confirmed Positive	Percentage Which Confirmed Is of Total Samples	Percentage Confirmed Is of Presumptive
March	411	64	35	9	55
April	350	89	77	22	87
May	432	39	12	3	31
June	424	121	58	14	48
July	285	84	42	15	50
August	487	186	172	35	92
September	278	55	27	10	49
October	330	83	48	15	58
November	275	32	21	8	66
December	281	41	37	13	90
Totals	3,553	794	529		67

The figures we have given for the Potomac water show the great variation in this relation under different conditions of pollution. The figures showing the occurrence of *B. coli* are the logical ones from which to judge of the sanitary condition of the water, and especially from which to compute efficiences of the various parts of the purification process, because they represent something much more definite than do the results of the presumptive tests. The latter, however, would be of value in the absence of the former, indicating with perhaps equal accuracy the variations in the polluted conditions of the water.

We have observed an interesting relation between the value of the presumptive test and the condition of the water under consideration. The condition of the water as regards pollution may be represented by the percentage which the number of samples proven to contain *B. coli* is of the total number of tests.

The value of the presumptive test for each month is given by the percentage of cultures determined as positive presumptive which are proven to contain *B. coli.*

These figures, when plotted, show a marked similarity in their variations, indicating that the value of the presumptive test as measured above varies with the quantity of pollution in the water, being least when the water is at its best, and greatest when there is the greatest quantity of pollution present in the water. This relation is indicated only for the particular class of water that comes under our routine examination, namely a surface water with only a moderate amount of pollution. Presumably the same relation would hold for any similar water not grossly and constantly polluted, but it would not hold for a water highly polluted with sewage.

(5) OTHER PRESUMPTIVE TESTS.

(*a*) *Direct plating on Wurtz litmus lactose agar plate.*—The Potomac water contains too little pollution for this method to give any satisfaction. The dextrose broth method is much more delicate, and we have discontinued attempts to get satisfactory indications by this means.

(*b*) *Neutral red reaction.*—Our trials of the neutral red reaction have given unreliable results. In these tests practically all the cultures confirmed positive have given the typical reduction of neutral red in the fermentation tube. The converse does not hold true. Of all the cultures giving typical reduction of neutral red, in conjunction with typical gas and CO_2 only 67 per cent were confirmed positive. There was one isolated case in which a negative neutral red corresponded to a negative confirmatory test. This condition is the principal one which should lend value to the test, and in the absence of any considerable number of samples fulfilling this condition, we can only conclude that the value of the test for application to this water is questionable.

(*c*) *Use of the bile salts.*—The use of the bile salts and their acids has been recommended for the preliminary cultivation in the examination of water for *B. coli.* We have been and are still making a study of the value of media of this kind as applied to the Potomac water. There is unquestionably a marked restraining influence exerted by the bile salts and their acids, upon the growth of bacteria, and unquestionably, too, this restraining influence is selective. The statement has been made that they *favored* the growth of *B. coli.* This statement seems ill advised, and we think should be qualified in accordance with the following views.

The restraining influence acts upon all forms of bacteria; in greatest degree upon water bacteria and saprophytes of that class; probably in the least degree upon *B. coli* and allied forms whose natural habitat is the intestine where they are exposed to somewhat similar conditions.

Our own figures, and also the figures given by others in this connection, indicate that under conditions of considerable pollution there are decided advantages in the use of lactose bile. But the water we have to examine here in our routine work, including not only the filtered water but also the unfiltered water during the greater part of the time, contains so little sewage pollution that a strong inhibiting agent cannot safely be used without running the risk of checking the development of the occasional sewage organisms along with the saprophytic forms.

We can say without hesitation that the data so far obtained during a three-months' period of trial of Jackson's "Bile Lactose," in comparison with dextrose broth, do not warrant us in giving up the latter in favor of the former.

The value of the test with the bile lactose on unpolluted or slightly polluted water, such as we have to deal with the greater part of the time, is uniformly less than with dextrose broth, except in the larger quantities of water.

The ideal media for preliminary cultivation is one that always permits and never inhibits the growth of *B. coli* while it does inhibit the growth of some other organisms, preventing in as large a measure as possible the effects of overgrowth and antagonism. The bile lactose has too great an inhibiting effect for our conditions and the dextrose broth too little. Therefore we may look with some hope of success to find a medium more nearly ideal for our conditions, by combining the good points of the two in proper proportions, and it is in this direction that we are now carrying our studies on this subject.

REVISION OF THE METHODS OF THIS LABORATORY FOR THE DETERMINATION OF *B. coli.*

A review of the foregoing data has led us to adopt the following scheme for the determination of *B. coli* in Potomac water:

(1) *Preliminary incubation in ordinary dextrose broth fermentation tubes at* 40° *C. for* 24 *hours.*—If no gas forms in 24 hours, the test may be considered negative. If gas forms within 24 hours, those tubes showing gas shall be fished, and litmus lactose agar plate cultures made. The quantity of gas, presence of CO_2, and reaction after incubation have no determinative significance, and therefore need not be recorded.

(2) *Litmus lactose agar plate.*—The litmus lactose agar plate shall be incubated at 40° C. for 18 to 24 hours, 18 being usually sufficient. Those plates showing no development of red colonies in 24 hours are considered negative. From those upon which red colonies have developed, representative red colonies shall be fished, and incubated upon agar slants.

(3) *Agar slants.*—Agar slants shall be incubated at 40° C. for 24 hours, and if retained for a longer period shall be kept in the 20° incubator. Agar slant cultures which have typical cultural characteristics need not be examined microscopically, Atypical cultures shall be examined in the hanging drop before deciding to discard or carry through subculture. This examination should be made within 24

hours, and not later than 48 hours. Within 48 hours the pure cultures which have passed the agar slant shall be inoculated into subculture media as follows:

(4*a*) *Dextrose broth fermentation tube.*—These shall be incubated at 40° C. for 24 hours when presence or absence of gas shall be noted. Absence of gas shall be recorded as negative. The quantity of gas, presence of CO_2, and reaction need not be recorded.

(4*b*) *Milk.*—This shall be incubated at 40° C. for two days, and examined daily for coagulation, and digestion of the casein. Coagulation by itself is positive. Failure to coagulate or digestion of casein after coagulation shall be recorded as negative.

(4*c*) *Nitrate broth.*—This shall be incubated, together with a blank, at 40° C. for two days and shall then be tested for nitrites If nitrites have been produced, the test is recorded as positive.

(4*d*) *Peptone broth.*—This shall be incubated together with a blank, at 40° C. for three days, and shall then be tested for indol. If indol has been produced, the test is recorded as positive.

On account of the insignificant value of the test for liquefaction of gelatin in the examination of the Potomac water, and the large quantity of glassware kept continually out of service for this test, it may hereafter be omitted from our routine examinations, without introducing any appreciable error.